EXHIBIT RAS-7

This is exhibit RAS-7 referred to in Declaration Under 37 C.F.R. 1.132 by Richard Anthony Strugnell dated 27.9.01

Richard Strugnell

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Production of Monospecific Antibodies to a Low-Abundance Hepatic Membrane Protein Using Nitrocellulose Immobilized Protein as Antigen

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Membrane proteins from primary cultures of rat hepatocytes were separated by two-dimensional polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose paper which was then dissolved in dimethyl sulfoxide and this mixture was used as a primary immunogen in rabbits. Subsequent immunizations were performed using nonsolubilized protein immobilized on nitrocellulose paper. A monospecific polyclonal antibody was generated against a specific mitochondrial membrane protein (MP-73) for which *de novo* synthesis appeared to be induced by amino acid starvation of the hepatocytes. A minimum of 15–20 μg of protein antigen was required to elicit significant antibody production. Serum antibody titer was sufficient to allow detection of MP-73 at a serum dilution of 1:2000. © 1987 Academic Press, Inc.

KEY WORDS: immunoblotting; membrane proteins; antibody production; mitochondria; hepatocytes; gel electrophoresis.

Antibodies have become a valuable tool in studies aimed at elucidating membrane protein topology, structure, and function. In addition, monospecific antibodies can provide the selectivity needed for further isolation and characterization of the membrane antigen. Often, a limitation in generating a monospecific antibody is the necessity to obtain a sufficient amount of the purified protein to use as immunogen. The majority of membrane proteins are of low abundance relative to the total protein content of the cell, so purification requires large amounts of starting material and is complicated by the insolubility of membrane proteins in aqueous solutions. In this report, we describe a methodology that allows the production of monospecific polyclonal antibodies following separation of complex mixtures of membrane proteins by two-dimensional polyacrylamide gel electrophoresis and electroblotting. Previous reports have demonstrated the utility of generating antibodies from polyacrylamide gel plugs containing a specific protein by either direct injection of the gel plug or extraction of the protein from the polyacrylamide prior to injection (1–3). Either of these procedures results in some loss of protein sample because of the handling procedures required. The methodology described in this report utilizes electrophoresed proteins that are transferred to nitrocellulose prior to immunization in order to concentrate the protein sample and to maximize the delivery of a low-abundance antigen. The nitrocellulose immobilized proteins are identified by Fast Green staining and the area of paper containing the specific protein of interest is excised from several replicate transfers. These pieces of nitrocellulose are then used as a solid-base immunogen.

MATERIALS AND METHODS

Materials. Nitrocellulose was purchased from Schleicher and Schuell. The minimal Eagle's medium (MEM) was obtained from Flow Laboratories and the ampholytes were

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purchased from LKB. All other materials and reagents were purchased from Sigma Chemical Co.

Cell culture. Rat hepatocytes were isolated from male Sprague–Dawley rats (120–140 g) by a collagenase perfusion technique described by Kilberg *et al.* (4). Hepatocyte populations exhibiting a cell viability greater than 90% were resuspended in culture medium and placed into primary culture (25×10^6 cells/150-mm culture dish). During the course of the experiment the cells were maintained under sterile conditions at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Isolation of a membrane-enriched fraction from cultured hepatocytes. Primary cultured hepatocytes were washed three times in phosphate-buffered saline, (150 mM NaCl, 10 mM sodium phosphate, pH 7.4, PBS)[2] and then incubated at 4°C for 5 min in a hypotonic buffer consisting of 1 mM sodium bicarbonate, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride (PMSF). The cells were removed from the culture dish with a rubber policeman and homogenized in a glass homogenizer with a tight-fitting Teflon pestle (100 strokes). The homogenate was centrifuged at 500*g* for 10 min to remove unbroken cells and nuclei, and the resulting supernatant was centrifuged at 10,000*g* for 30 min. The membrane pellet was resuspended in hypotonic buffer and stored at −70°C. Typically, 1–2 mg protein per 25×10^6 hepatocytes was recovered in the membrane pellet.

Two-dimensional polyacrylamide gel electrophoresis. Hepatic membrane proteins were solubilized by the alkaline–urea method of Horst *et al.* (5). In brief, membrane proteins (1–2 mg) were susp 900 μl of 5 mM K_2CO_3 containing 9 (pH 10.3) for 5 min. The solutio luted with Nonidet-P40 (NP-40) a threitol to final concentrations o 0.5%, respectively. Approximately solubilized membrane protein was plied to the cathode end of an isoe cusing polyacrylamide gel contain acrylamide, 0.85% diallyltartardia M urea, 2% NP-40, and 2% amphol ing from pH 3.5 to 11.0 and focus anode (75 V for 0.5 h; 150 V for 2 for 17 h; 450 V for 3 h). After foc gel was equilibrated for 10 min i Tris–HCl, pH 6.9, 1% sodium do fate (SDS), and 1% 2-mercaptoeth proteins were then subjected to elec sis in the second dimension usin polyacrylamide–SDS system simil described by Laemmli (6). Followi mensional polyacrylamide gel elec sis (2D-PAGE), the proteins wer phoretically transferred to nitroce described by Towbin *et al.* (7).

Antibody production. The followi odology is a modification of a pub port by Knudsen (8). Membrane p interest were separated by 2D-P electrophoretically transferred to n lose. The proteins were localized b the nitrocellulose paper with 1% F dye (5 min) and destained in nol:water:acetic acid (50:40:10, same protein spot was excised fro replicate nitrocellulose blots (6–10 and dissolved in dimethyl sulfoxide as described by Knudsen (8). An ume of Freund's adjuvant was add DMSO mixture; the solution was and then injected subcutaneousl sites along the back of a male Ne white rabbit.

All subsequent secondary immu were performed by implanting neously nonsolubilized protein im on nitrocellulose (T. W. O'Brien, monaco, and M. Bryant, submitte

[2] Abbreviations used: PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; NP-40, Nonidet-P40; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; ASN, L-asparagine; ACT, actinomycin D; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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lication). The protein spot was excised from Fast Green stained nitrocellulose blots, rolled into the shape of a cylinder, and inserted into the bore of a 16-gauge hypodermic needle (16 × 1 ½). The nitrocellulose was implanted in the rabbit by expelling it with a stainless-steel rod. The rabbit was bled from a lateral ear vein 10 days following each secondary (boost) implantation and antibody production was measured using immunoblotting techniques.

Detection of protein antigens immobilized on nitrocellulose. Hepatic membrane proteins were separated by 1D- or 2D-PAGE and electrophoretically transferred to nitrocellulose as described by Towbin *et al.* (7). Free binding sites on the nitrocellulose paper were then blocked by soaking them for 2–4 h in PBS containing 3% bovine serum albumin (BSA), 0.5% Tween 20, and 0.01% NaN_3 (blot buffer). Antisera obtained from immunized rabbits was diluted appropriately in blot buffer and incubated with the nitrocellulose paper for 2 h at 25°C. The blot was then washed for 1 h with several changes of PBS containing 1% BSA and 0.5% SDS. Bound antibody was detected by incubating the nitrocellulose with ^{125}I-labeled Protein A (1×10^6 cpm/ml blot buffer). After 60–90 min, unbound ^{125}I-labeled Protein A was removed by repeated washing of the nitrocellulose paper in PBS containing 1% BSA and 0.5% SDS for 1 h. The bound ^{125}I-labeled Protein A was visualized by autoradiography approximately 12 h).

RESULTS AND DISCUSSION

Regulation of gene transcription by small nutrient molecules represents an interesting phenomenon with regard to cellular regulation of metabolism. The goal of the present research was to devise a procedure for the preparation of antibodies against specific proteins in order to study the effect of amino acid deprivation of cultured cells on the transcriptional regulation of individual membrane proteins. We have identified several membrane proteins for which synthesis appears to be induced during periods of amino acid starvation. Figure 1 shows the results of a series of experiments in which primary cultured rat hepatocytes were incubated with [^{3}H]leucine under conditions of either amino acid deprivation (KRB, Krebs-Ringer bicarbonate buffer), amino acid deprivation in the presence of actinomycin D (KRB containing 10 μM actinomycin D), or amino acid supplementation (KRB containing 20 mM L-asparagine). Following homogenization, the membrane proteins in a crude membrane fraction were analyzed by 2D-PAGE, Coomassie blue stained, and subjected to fluorography. Radiolabeled-leucine incorporation was determined for each protein as described in the legend to Fig. 1 and the results were expressed as a ratio of [^{3}H]leucine incorporation comparing amino acid deprivation to amino acid supplementation (cpm KRB/cpm KRB containing 20 mM L-asparagine) or as a ratio of [^{3}H]leucine incorporation during amino acid deprivation in the presence or absence of actinomycin D (cpm KRB/cpm KRB containing 10 μM actinomycin D). As seen in Fig. 1A, the synthesis of five selected proteins is enhanced in hepatocytes incubated in amino acid-free medium. Of these, the induction of a protein (MP-73) corresponding to an isoelectric point of 7.0 and a molecular mass of approximately 73 kDa was shown to be sensitive to inhibition of RNA biosynthesis. Additional studies involving pulse-chase labeling protocols have suggested that the amino acid-dependent biosynthesis of MP-73 is regulated at the transcriptional level (9).

Monospecific antibodies directed against this protein would represent an important tool to investigate its regulation and biosynthesis. Based on Fast Green staining of a nitrocellulose blot containing 300 μg of membrane protein, the estimated content of MP-73 represents approximately 0.4 ± 0.1% of the membrane proteins in the fraction under study. Purification of the protein and production of antisera using conventional

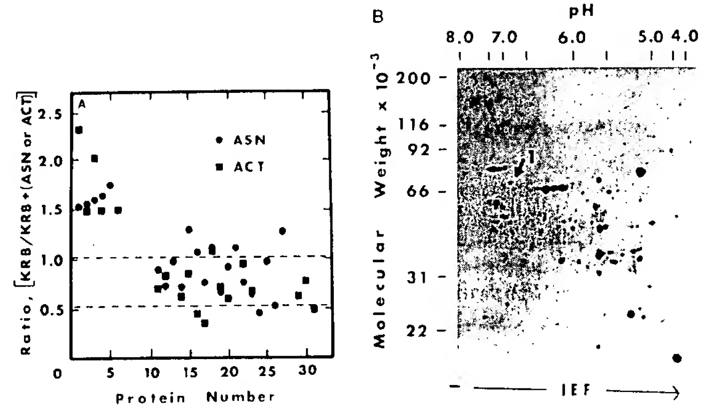

FIG. 1. Synthesis of individual hepatic membrane proteins. Rat hepatocytes were isolated a into primary culture (25×10^6 cells/150-mm petri dish) in either KRB or KRB containin L-asparagine (ASN), or KRB containing 10 μM actinomycin D (ACT). All of the above c contained 40 μCi/ml [^{3}H]leucine and the cells were incubated at 37°C for 6 h. A crude membran (10,000*g* pellet) was isolated as described under Materials and Methods. The membrane pro then subjected to two-dimensional polyacrylamide gel electrophoresis followed by fluorogra proteins of interest were excised as gel plugs and the radioactivity in each was quantitated by sc spectrometry. The results are expressed as the ratios of cpm (KRB)/cpm (KRB + ASN) or cp cpm (KRB + ACT) incorporated into individual proteins (A). Protein MP-73, marked as spot (B), was used to prepare monospecific polyclonal antibodies.

methodology would be hampered by the low abundance of MP-73 in our membrane fraction. Production of antibodies against the protein following 2D-PAGE would solve the dilemma by providing, in effect, a one-step purification. Furthermore, through transfer of the protein to nitrocellulose prior to immunization one might maximize the amount of protein presented to the rabbit.

Initially, nitrocellulose-bound antigen was dissolved in DMSO as described by Knudsen (8). To estimate the amount of protein used for the immunizations, a standard curve was prepared by spotting 1–10 μg bovine serum albumin on nitrocellulose and then staining the paper with 1% Fast Green. The staining intensities of the excised MP-73 spots were compared to this standard curve. Six protein-bearing spots corresponding to approximately 7 μg of MP-73 protein were excised from a series of six two-dimensional polyacrylamide gels following electrophoretic transfer of the proteins to ni paper. The piece of nitrocellulo MP-73 was bound was dissolve mal volume of DMSO, an equa Freunds's complete adjuvant wa then this mixture was used as immunogen. More recently we nated the DMSO solubilization s ply implanted the nitrocellulos cutaneously following wetting w complete adjuvant. Four weeks mary injection, the following in schedule was followed by impla taneously nonsolubilized nitro mobilized protein as a solid ph gen: Day 1, 8 protein spots (10 10 protein spots (10 μg); Day 2 spots (10 μg).

The time course of antibody as monitored by one-dimensio blotting, is shown in Fig. 2. T the second set of implantations

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antibody production was detected readily. Antibody levels were maintained following two additional implantations, each spaced at 2-week intervals. Approximately 4 weeks following the final injection of antigen, antibody was no longer detectable (Fig. 2).

The serum antibody titer, tested after the third injection (second boost) of antigen, was sufficient to allow immunoblotting to be routinely performed at dilutions ranging from 1:250 to 1:500 (Fig. 3). The same antibody mixture, diluted 1:250, could be used

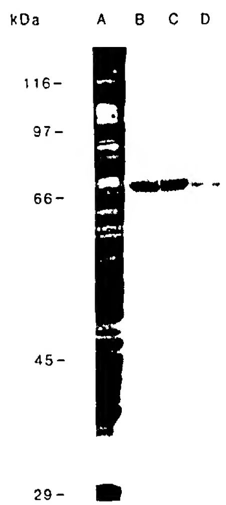

FIG. 2. Time course of antibody production. A New Zealand white rabbit was immunized as described in the text. Four weeks following the primary implantation of nitrocellulose spots, the rabbit was boosted at 2-week intervals and bled 10 days after each boost. Serum was assayed for antibody production (1:50 dilution) using an immunoblotting procedure against a 10,000g crude membrane fraction separated by one-dimensional polyacrylamide gel electrophoresis and then transferred to nitrocellulose (25 μg protein/lane). Bound antibody was detected using ^{125}I-labeled Protein A as described in the text. Lane A represents total protein of the membrane fraction stained with amido black, while Lanes B, C, and D are immunoblots designed to test for the presence of antibody at 6 (B), 8 (C), and 10 (D) weeks after the primary injection. Lanes E, F, and G show the antibody level at 4, 6, and 8 weeks following the last set of boost implantations. Lane H illustrates the reaction seen with preimmune serum.

up to five times for immunoblotting without noticeable loss of immunoreactivity. Indeed, as might be expected, the amount of nonspecific binding decreased with each use and can be decreased by preabsorbing the antibody mixture in the presence of nitrocellulose paper that has been incubated previously in blotting buffer.

As mentioned above, the DMSO solubilization step, used to prepare the immunogen in our first series of experiments, is not necessary. The use of nonsolubilized protein as a primary antigen by simply implanting the nitrocellulose paper subcutaneously greatly minimizes the loss of antigen which occurs during the DMSO solubilization and injection procedure. Furthermore, we have found that some proteins will elicit antibody production without the use of Freund's complete adjuvant. For those proteins that do not, the nitrocellulose spots used to boost a second time (according to the schedule above) can be wetted with complete adjuvant. In most cases, these procedures result in an acceptable antibody titer. Production of antisera in this manner, without adjuvant, may be useful for bacterial antigens.

The specificity of the antisera against MP-73 was examined by adapting the immunoblotting technique to an analysis of membrane proteins separated by 2D-PAGE (Fig. 4). The position of the immunoreactive protein coincides with that of the antigen previously observed by Coomassie blue staining (i.e., pI 7.0, M_r 73 kDa). The immunoreactive polypeptide at lower molecular mass (47 kDa) seen in Fig. 4 is not present consistently and is not detected by preimmune serum. This polypeptide apparently represents a proteolytic cleavage product contaminating some membrane preparations. The monospecific antiserum was also used to determine the subcellular location of the antigen. This was achieved by subcellular fractionation of rat liver. Fractions enriched in cytoplasm, plasma membrane, golgi, endoplasmic reticulum, and mitochondria were subjected to 1D-PAGE, the proteins

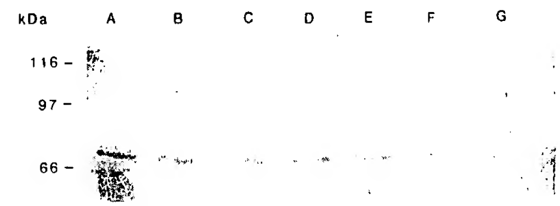

FIG. 3. Serum antibody titer. Membrane proteins (50 μg/lane) were separated by one-dimen electrophoresis, electrophoretically transferred to nitrocellulose, and then subjected to immunob described under Materials and Methods. Serum obtained after the third injection (second boost a after the primary injection) was assayed by immunoblotting using the following dilutions: Lane Lane B (1:75), Lane C (1:100), Lane D (1:300), Lane E (1:500), Lane F (1:1000), and Lane G Bound antibody was detected with ^{125}I-labeled Protein A.

were then electrophoretically transferred to nitrocellulose and immunoblotted with anti-MP-73 as described above. Only the mitochondrial-enriched fraction contained detectable amounts of the MP-73 protein (data not shown).

Collectively, these results indicate that monospecific antibody production can be elicited using less than 20 μg protein through the use of antigen that is concentrated and immobilized on nitrocellulose. The proce-

dure can be readily applied to s are obtainable only in small qua as proteins present in cells maint sue culture. As shown here, an feature is the ability to prepare against membrane-bound protei difficult to solubilize and purify. methodology does not require pr cation beyond identification of th a two-dimensional gel electrop tern. Separation of proteins by

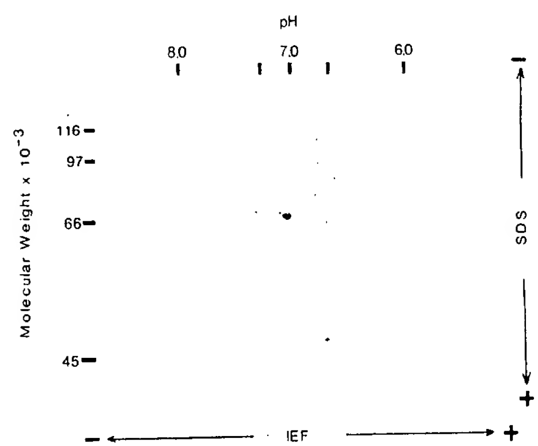

FIG. 4. Two-dimensional immunoblot. Membrane proteins (300 μg) were separated by two-din polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Serum after the second boost (8 weeks after the primary injection) was diluted 1:250 and used to detect t protein by the immunoblotting procedures described in the text.

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rather than by one-dimensional gel electrophoresis prior to immunization greatly enhances the probability of obtaining a monospecific antibody and the use of nitrocellulose-bound protein as a solid-phase immunogen results in nearly a 100% delivery of the antigen. The method presented should allow production of antibodies against proteins that can be identified on a two-dimensional gel or blot by procedures such as enzymatic activity, ligand binding, or selective covalent modification.

ACKNOWLEDGMENTS

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Separation of Human from Mouse and Monkey Adenosine Deaminase by Ion-Exchange Chromatography following Retroviral-Mediated Gene Transfer

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A method for the chromatographic separation of human adenosine deaminase (ADA) fron murine and monkey ADA is described. This procedure was developed in order to detect th expression of low or moderate levels of human ADA following retroviral-mediated gene transfe of cloned human ADA gene sequences into both mouse and monkey cells. Protein separatioi was achieved on a Mono Q (HR 5/5) anion-exchange column using the Pharmacia fast proteii liquid chromatography system and was found to be a highly reproducible method yieldin enzymatically active protein. An increasing linear gradient extending from 0.05 to 0.5 M potassium chloride (pH 7.5) was used to elute the enzyme. Under these conditions, most humai ADA does not bind to the column and elutes in the low-salt buffer (0.05 M KCl), while murin ADA elutes at 0.12 M KCl and monkey ADA at 0.15 M KCl. The column fractions were assayec for ADA activity, and the characteristic isozyme banding patterns for human, mouse, anc monkey ADA were confirmed by starch gel electrophoresis. This procedure allows the rapid anc reproducible separation of human ADA from that of other species and yields partially purifiec enzymatically active protein. © 1987 Academic Press, Inc.

KEY WORDS: adenosine deaminase; ion-exchange chromatography; FPLC; TLC.

Adenosine deaminase (ADA[1]; EC 3.5.4.4) is an important enzyme of purine metabolism, catalyzing the deamination of adenosine and deoxyadenosine (1). In man, an absence of ADA activity is associated with one form of severe combined immunodeficiency disease (ADA$^-$ SCID; reviewed in (2,3)). The ADA protein has been identified and characterized from a variety of sources including amphibians (4,5), birds (6), and mammals (7–12). In humans, ADA is a ubiquitous protein with several isozymes that their electrophoretic mobilities (Human red blood cell (RBC) ADA a characteristic triple-banded patte: analyzed by starch gel electrophoı while ADA from other tissues is com isozymes which do not migrate tov anode as far as RBC ADA (13). erythrocyte ADA also exhibits multi trophoretic forms by isoelectric focu: corresponding to a single polypeptic with an approximate molecular wei; of 38,000 (17) which has undergone degrees of glycoslyation (16). A larg of human ADA (M_r 298,000) has b lated from several tissues (15,18) an mined to be a complex of the 38, ADA species and a larger binding (M_r 200,000). The large form is predc in tissues which exhibit lower ADA

[1] Abbreviations used: ADA, adenosine deaminase; DCF, 2'-deoxycoformycin; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: Thiazolyl blue); RBC, red blood cell; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; LSM, Lymphocyte Separation Medium; Buffer A, 0.05 M potassium chloride, 20 mM Tris-HCl, pH 7.5; Buffer B, 1.0 M KCl, 20 mM Tris-HCl, pH 7.5.

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